Seminal and Nodal Roots of Barley Differ in Anatomy, Proteome and Nitrate Uptake Capacity

Zhaojun Liu1, Ricardo Fabiano Hetfwer Giehl1, Anja Hartmann1, Mohammad Reza Hajirezaei1, Sebastien Carpentier2,3 and Nicolaus von Wirén1,*

1Molecular Plant Nutrition, Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, 06466 Gatersleben, Germany
2Proteomics Core Facility, SYBIOMA, KU Leuven, O&N II Herestraat 49, Bus 901, 3000 Leuven, Belgium
3Division of Crop Biotechnics, Department of Biosystems, KU Leuven, Willem de Croylaan 42, Box 2455, 3001 Leuven, Belgium
*Corresponding author: E-mail, vonwiren@ipk-gatersleben; Fax: +49 39482 5135.
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The root system of barley plants is composed of embryonic, seminal roots as well as lateral and nodal roots that are formed postembryonically from seminal roots and from the basal part of shoots, respectively. Due to their distinct developmental origin, seminal and nodal roots may differ in function during plant development; however, a clear comparison between these two root types has not yet been undertaken. In this study, anatomical, proteomic and physiological traits were compared between seminal and nodal roots of similar developmental stages. Nodal roots have larger diameter, larger metaxylem area and a larger number of metaxylem vessels than seminal roots. Proteome profiling uncovered a set of root-type-specific proteins, including proteins related to the cell wall and cytoskeleton organization, which could potentially be implicated with differential metaxylem development. We also found that nodal roots have higher levels of auxin, which is known to trigger metaxylem development. At millimolar nitrate supply, nodal roots had approximately 2-fold higher nitrate uptake and root-to-shoot translocation capacities than seminal roots, whereas no differences were found at micromolar nitrate supply. Since these marked differences were not reflected by the transcript levels of low-affinity nitrate transporter genes, we hypothesize that the larger metaxylem volume of nodal roots enhances predominantly the low-affinity uptake and translocation capacities of nutrients that are transported with the bulk flow of water, like nitrate.

Keywords: Hordeum vulgare • Nitrogen translocation • Nitrate transport • Proteomics • Root development • Xylem development.

Introduction

Plant roots serve important functions in plant development and fitness as they represent the main entrance of water and nutrients from the soil into the plant (Marschner 2012). The root system of barley plants consists of embryonic and post-embryonic roots. Embryonic roots, referred to as seminal roots in graminaceous species, are formed during embryogenesis and, in barley, range in number between 4 and 8 (Jia et al. 2019). Postembryonic roots are formed after germination and include lateral roots, which emerge from seminal roots, and a class of shoot-borne roots called nodal roots (or crown/brace roots), which develop from the basal part of the shoot (Rogers and Benfey 2015). It has been proposed that seminal roots are important for plant establishment at early developmental stages, while nodal roots become more important at later stages when they dominate the root system of adult cereal plants (Hochholdinger et al. 2018). A very early study with hydroponically cultured wheat, barley and rye plants has indicated that seminal roots only absorb 25% of the water between flowering and maturity, while the other 75% of the water was absorbed via nodal roots (Krassovsky 1926).

Although in a particular species, seminal and nodal roots share the same genetic background, increasing evidence shows that they differ in their anatomy and physiology. At the anatomical level, nodal/crown roots of cereals are generally thicker, with a wider central stele (Krassovsky 1926) and, in maize and pearl millet, tend to have larger metaxylem areas compared with seminal roots (Yu et al. 2015a, Passot et al. 2016). When exposed to hypoxia, wheat can form a 30% larger aerenchyma area in nodal roots compared with seminal roots, suggesting that nodal roots have higher anatomical plasticity and might be more efficient for O2 transportation under hypoxia (Thomson et al. 1990). At the physiological level, maize crown roots showed higher hydraulic conductivity (Tai et al. 2016) and water uptake capacity (Ahmed et al. 2018) as compared to seminal roots. In the case of millet and sorghum, nodal roots of millet were more plastic, i.e. formed earlier nodal roots in response to drought stress (Rostamza et al. 2013). However, to what extent these anatomical and physiological differences influence the function of such root types has not yet been investigated.

One of the important functions of plant roots is nutrient uptake to sustain plant metabolism. A functional–structural root simulation approach has predicted that nitrate uptake...
can be increased in maize by decreasing the density and increasing the length of lateral roots (Postma et al. 2014). Furthermore, maize brace roots, which originate from aboveground shoot nodes, have been shown to respond more strongly than seminal roots to locally supplied nitrate (Yu et al. 2016). This suggests that postembryonic root type has a superior morphological plasticity allowing for more efficient adaptation to external nitrogen (N) availability. Moreover, a field study in six environments with 10–67 maize cultivars showed that grain yield correlated positively with crown root number and length under both high and low N treatments (Liu et al. 2019a). Thus, it appears that increased crown root formation contributes to efficient N uptake and finally to higher yield. However, it has not yet been directly assessed whether seminal and nodal roots exhibit differential N uptake capacities.

In the present study, we investigated in barley the different properties of seminal and nodal roots of similar developmental stage. By concomitant analysis of anatomical and physiological traits, we observed that nodal roots have a larger metaxylem area that is associated with a higher capacity for nitrate uptake and translocation. Our results suggest that the number and area of metaxylem vessels in nodal roots could represent interesting targets when selecting genotypes for higher nitrogen uptake and translocation efficiencies.

Results

Anatomical traits in seminal and nodal roots

To assess developmental and anatomical features of seminal and nodal roots, we cultivated barley plants (cv. Golden Promise) in hydroponics with full nutrient supply. The first seminal root emerged from the basal pole of the seed 4 d after sowing (Fig. 1A). Three days later, two additional seminal roots were visible (Fig. 1B), and after 10 d, a final number of six seminal roots had appeared, which is typical for this cultivar. Each seminal root formed a slim body of approximately 400 µm diameter with 4–5 cortical cell layers and one large central xylem vessel (Fig. 1C, D). The first nodal roots appeared only 20 d after sowing (Fig. 1E, F), and their number increased steadily throughout the experiment. When we compared the mature zone of seminal and nodal roots, we observed that nodal roots were visibly thicker than seminal roots (Fig. 1C–G). Upon quantification, we determined that the average diameter at the mature zone of nodal roots was ~20% greater than that of seminal roots (Fig. 1I).

Cross-sections of the mature root zone of seminal and nodal roots revealed that also nodal roots exhibited a single layer of epidermal cells of similar total cell area (Fig. 1H, J). Furthermore, in agreement with previous reports on the barley cultivars Morex and Golf (Knipfer and Fricke 2011, Kirschner et al. 2017), cross-sections of the mature root zone of cv. Golden Promise showed that nodal roots contained five to six layers of cortical cells and thus in average one cell layer more than seminal roots (Fig. 1D, H). Although cortical cells of nodal roots tended to be larger than those of seminal roots, there was no significant difference in the area of this cell layer between both root types (Fig. 1I). Instead, the most striking anatomical difference was observed in the central cylinder where nodal roots contained up to three central metaxylem vessels and as many as 10 smaller peripheral vessels, whereas in seminal roots one central metaxylem vessel was surrounded by eight small peripheral vessels (Fig. 1D, K). Thus, seminal and nodal roots in barley differ not only in developmental origin but also in their anatomical structure, especially regarding the number of metaxylem vessels in the central cylinder.

Seminal and nodal roots differ in their proteome

To identify major molecular determinants underlying the observed anatomical differences in seminal and nodal roots, we performed a proteome analysis. We followed the procedure described by Tai et al. (2016) and harvested seminal and nodal roots of approximately the same length (i.e. 50–60 cm length from the root apex). To allow the comparison of root types at similar developmental stage, these samples were collected at 25 d after sowing for seminal roots and 39 d after sowing for nodal roots. Extracted proteins were digested and subjected to proteome profiling via ultra-high-pressure liquid chromatography–mass spectrometry (UPLC-MS/MS). In total, we were able to identify in at least two out of five biological replicates, 1,188 proteins in seminal roots and 1,160 in nodal roots (Supplementary Table S1 and S2). A principal component analysis of the protein profiles allowed us to distinguish seminal and nodal root samples in particular along PC1, which explained 51.4% of the overall observed variation (Fig. 2A). Although 75%, i.e. 904 proteins, of the detected proteins were present in both root types, 284 and 256 proteins were identified only in seminal or nodal roots, respectively (Fig. 2B). In addition, 159 and 67 proteins were significantly more abundant, i.e. $P < 0.05$ and $\log_2(\text{fold change}) > 1$, in seminal or nodal roots, respectively. These numbers pointed to substantial differences in the expressed protein profiles of seminal and nodal roots.

We then combined proteins identified exclusively in one root type together with those being significantly more abundant in the same root type, which resulted in 443 and 323 proteins in seminal and nodal roots, respectively (Fig. 2B, Supplementary Tables S2 and S3). A gene ontology (GO) analysis showed that 18 GO terms were significantly enriched in seminal roots, while the corresponding number was 14 in nodal roots. Interestingly, although there was no protein identifier shared between these two protein groups, 12 GO terms were commonly enriched between seminal and nodal roots (Supplementary Figs. S1, S2).

To further obtain more detailed information of these unique or preferentially expressed proteins in each root type, we employed a MapMan-based Mercator-automated sequence annotation pipeline (Lohse et al. 2014). Annotation categorized these two input protein groups into 27 bins, which covered diverse biological functions in plants (Fig. 2C). Although these two protein inputs differed in abundance between seminal and nodal root types, there was a large overlap in the identified functional categories. However, the number of proteins belonging to each category differed substantially when comparing seminal with nodal roots (Fig. 2C). There were a larger number...
of proteins from seminal roots in categories like ‘lipid metabolism’ and ‘enzyme classification’. By contrast, proteins belonging to ‘RNA processing’ showed larger numbers in nodal compared with seminal roots (Fig. 2C). Since we observed significant anatomical differences between seminal and nodal roots (Fig. 1), we were especially interested in the bins ‘cytoskeleton organization’ and ‘cell wall organization’, because proteins belonging to these bins are involved with plant cell morphogenesis (Barrero et al. 2002, Szymanski and Cosgrove 2009). There were 14 or 6 proteins belonging to the bin ‘cytoskeleton organization’ in seminal or nodal roots, respectively (Fig. 2D, Supplementary Table S5). Most of these proteins belong to actin or tubulin protein families, suggesting the existence of a root-type-specific abundance of these protein families. For example, kinesin-7 motor (protein ID: f2e8d0) was only identified in seminal roots, while SPIRAL1-like microtubule-stabilizing factor (protein ID: f2e2m2) was present specifically in nodal roots (Fig. 2D). In the bin ‘cell wall organization’, two or four proteins were more abundant in seminal or nodal roots, respectively (Fig. 2D, Supplementary Table S5). Among them, pectin acetyl-xylan esterase 5-like (protein ID: f2d4a1) and coumarate 3-hydroxylase (protein ID: f2d6v0) showed high abundance in seminal or nodal roots, respectively. Members of the pectin acetyl-xylan esterase family play an important role in cell elongation due to their functions in modulating extensibility of the cell wall (Gou et al. 2012) while coumarate 3-hydroxylase family proteins are
essential for the biosynthesis of lignin, also during xylem lignification (Ralph et al. 2006). A flavonoid O-methyltransferase (protein ID: a5ytr4) was also identified in seminal roots. Proteins like endoglucanase 12 (protein ID: m0vm74), cellulose synthase interactive 1 (CSI1) (protein ID: m0w2v6) and cytochrome b5 (protein ID: f2dal6) were identified in nodal roots.
Taken together, our proteome profiles revealed that seminal and nodal roots differ predominantly in proteins belonging to the bins ‘cell wall organization’ and ‘cytoskeleton organization’.

**Cytokinin and auxin analysis**

Since xylem differentiation is largely dependent on the interaction between cytokinins and auxin (Bishopp et al. 2011, De Rybel et al. 2014), we assessed the levels of these plant hormones in both seminal and nodal roots via UPLC–MS/MS. The physiologically active cytokinin forms trans-zeatin, N\(^6\)-(\(\Delta\) 2-isopentenyl) adenine and cis-zeatin were below the detection limits of our analytical method and could not be assessed. The levels of N\(^6\)-(\(\Delta\) 2-isopentenyl) adenine riboside, cis-zeatin riboside and trans-zeatin riboside, which are the major transport forms of cytokinins (Hirose et al. 2007), were not significantly different in seminal and nodal roots (Fig. 3A). However, we found that nodal roots contained 20% or 30% higher concentrations than seminal roots of the physiologically most active auxin form IAA and of its conjugated form methyl-IAA, respectively (Fig. 3B). The levels of the inactivated auxin form oxindole-3-acetic acid did not differ significantly between both root types (Fig. 3B). These higher IAA levels in nodal roots may relate to the role of auxin in root development or in physiological processes.

**Nitrate uptake and translocation differ in seminal and nodal roots**

The distinct features of seminal and nodal roots observed at multiple levels prompted us to investigate whether these two root types also exhibit functional differences, such as in nutrient uptake. Therefore, seminal and nodal roots were guided separately into cylinders containing low or high concentrations of \(^{15}\)N-labeled nitrate for either short- or mid-term uptake assays (Supplementary Fig. S3). When exposed to 200 \(\mu\)M nitrate for 5 min, \(^{15}\)N influx was not significantly different between the two root types (Fig. 4A), suggesting that seminal and nodal roots have similar high-affinity nitrate uptake capacities. This result also held true, when the calculation of nitrate uptake rates was referred to unit root length instead of root dry mass (Supplementary Fig. S4A). Interestingly, when exposed to a solution containing 4 mM nitrate for 2 h, nodal roots exhibited almost 2-fold higher low-affinity nitrate uptake capacity as compared with seminal roots (Fig. 4B). This difference was even more striking when \(^{15}\)N uptake rates were normalized to unit root length (Supplementary Fig. S4B). A longer uptake period was chosen in the low-affinity range to allow determining root-to-shoot translocation rates, which turned out to be 2-fold higher in nodal roots (Fig. 4C, Supplementary Fig. S4C). Since nitrate uptake in roots strongly relies on the supply of assimilates from shoots (Lejay et al. 2003), we assessed sugar levels in both root types. Our results showed that nodal roots had 40% higher sucrose concentrations, whereas the levels of glucose and fructose were comparable in both root types (Fig. 4D). In a separate experiment, we examined root-type-specific translocation rates of two nonessential elements that are widely used as tracers, namely Rb\(^{\text{1}}\) for K\(^{\text{1}}\) and Sr\(^{2+}\) for Ca\(^{2+}\) (Gierth et al. 2005, Duan et al. 2018). In absolute terms, translocation rates appeared to be approximately 10 times lower for Rb\(^{\text{1}}\) and approximately 100 times lower for Sr\(^{2+}\) (Supplementary Fig. S5) than for nitrate (Fig. 4C). Translocation rates of Rb\(^{\text{1}}\) were not significantly different between root types but just tended to be higher in nodal than in seminal roots, while translocation rates of Sr\(^{2+}\) were highly similar in both root types. Thus, the larger xylem diameter in nodal roots had no significant impact on these two mineral elements with lower translocation rates. Taken together, our results revealed that seminal and nodal roots do not differ in their high-affinity nitrate uptake capacity, whereas nodal roots exhibit a higher low-affinity nitrate uptake capacity than seminal roots, which also relates to a higher root-to-shoot N translocation capacity.

**Expression levels of nitrate transporter genes in seminal and nodal roots**

To investigate whether the enhanced low-affinity nitrate uptake capacity of nodal roots was due to differential expression of
nitrate transporters involved with nitrate uptake or xylem loading, we assessed the expression of these genes by quantitative real-time-PCR. We used the IPK barley BLAST server (https://webblast.ipk-gatersleben.de/barley_ibsc/) to identify all accessible genes from the NRT1 family in barley. The corresponding amino acid sequences were then subjected to phylogenetic analysis together with NRT1.1, NRT1.2 and NRT1.5 proteins from rice (Supplementary Fig. S6). We focused on these three NRT families as they represent the major low-affinity nitrate transporters and nitrate xylem loaders in plants (Orsel et al. 2002, Lin et al. 2008). The phylogenetic analysis revealed that barley NRT1.1, NRT1.2 and NRT1.5 families contain 4, 2 and 3 genes, respectively (Supplementary Fig. S6). Among these nine genes, the putative NRT1.1 orthologs HORVU7HR1G071600, HORVU4HR1G088020, HORVU1HR1G053990 and HORVU1HR1G054020 as well as the putative NRT1.5 ortholog HORVU6HR1G066840 exhibit relatively high expression in root tissues according to the expression data deposited in the IPK barley database.

Our qRT-PCR analysis showed that the expression levels of genes from the NRT1.5 family were highly similar in seminal and nodal roots (Fig. 5A). Among the putative NRT1.1 and NRT1.2 homologs, only the expression of one of the NRT1.1 genes, HORVU7HR1G071600, was ~30% higher in nodal roots than in seminal roots (Fig. 5B). The expression levels of other members of the NRT1.1 and NRT1.2 families did not differ significantly in the two root types (Fig. 5B). Together, these data indicated that differences in expression levels of genes encoding transporters for low-affinity uptake and xylem loading of nitrate make only a small contribution to the marked difference in nitrate uptake and translocation between seminal and nodal roots.

**Discussion**

**Barley seminal and nodal roots exhibit distinct anatomy, auxin levels and proteome profiles**

About one century ago, it had been documented that nodal roots of wheat, barley and rye are generally thicker than seminal roots (Krassovsky 1926). A few subsequent studies in these and other graminaceous species have shown that seminal and nodal roots differ in their development, especially in morphological plasticity, which became particularly evident when these root types were exposed to different stresses (Rostamza et al. 2013, Yu et al. 2015a, Passot et al. 2016, Liu et al. 2019b). In the present study, we investigated whether in barley the significantly larger metaxylem of nodal roots relates to a higher capacity for low-affinity nitrate uptake and translocation.

In barley, structural differences between seminal and nodal roots relied clearly on a larger xylem area in nodal roots, which...
was due to a larger number of central metaxylem vessels (Fig. 1). Larger numbers of metaxylem vessels in nodal roots also hold true in other graminaceous species. For example, crown roots of maize inbred line B73 have by average 11 metaxylem vessels while seminal roots contain only five (Tai et al. 2016). In case of pearl millet, the inbred lines originating from Indian, West and Central African landraces have three metaxylem vessels in nodal roots compared with one in seminal roots (Passot et al. 2016). Crown roots of maize also have larger diameter as well as cortical cell areas compared with seminal roots (Yu et al. 2015a). The molecular mechanism that causes these anatomical differences between root types in graminaceous species is still unknown. However, in Arabidopsis, a mutation in the NAC (NAM, ATAF1/2, and CUC2) transcription factor XYLEM NAC DOMAIN 1 (XND1), which antagonizes xylem differentiation by negatively regulating secondary cell wall synthesis and programmed cell death (Zhao et al. 2007), resulted in enlarged xylem vessel number and area and increased root hydraulic conductivity (Tang et al. 2018). This suggests that metaxylem vessel number and size can affect particular root functions.

In addition to the differences in anatomy, our results show that barley seminal and nodal roots also differ in their proteome
(Fig. 2A). Previously, it had been shown that different root types of maize can be distinguished based on their transcriptome profiles (Tai et al. 2016). Crown and seminal roots did not only show distinct functional categories, such as cell organization and cell wall biosynthesis, but also differential enrichment of transcription factors from various families; in particular, NAC, WRKY and bHLH (basic Helix-Loop-Helix) transcription factors were more enriched in seminal roots compared with nodal roots. In *Medicago truncatula*, several members of these families, such as MtNST1 (Zhao et al. 2010) and MtSTP (Wang et al. 2010), are involved in cell wall organization, while mutation of the bHLH transcription factor LONESOME HIGHWAY of Arabidopsis results in the reduction of 50% metaxylem cells compared with wild-type plants (Ohashi-Ito and Bergmann 2007). The findings of Tai et al. (2016) and those presented herein suggest that anatomical differences between seminal and nodal roots are, at least in part, due to the expression of different cell wall developmental programs in these two root types. Interestingly, functional categorization of the protein profiles in our study revealed root-type-specific abundance of several cell wall- and cytoskeleton-related proteins, such as actin and tubulin protein isoforms. Most of those proteins were more abundant in seminal roots (Fig. 2C, D, Supplementary Table S5). Also, in the study of Tai et al. (2016), alpha tubulin 4, alpha tubulin 6, beta tubulin 2 and beta tubulin 4 showed higher transcript levels in primary roots compared with crown roots of maize. In eukaryotic cells, actin and tubulin proteins polymerize to form cytoskeletal filaments that maintain cell shape and internal structural organization (Oda and Hasezawa 2006). During xylem development, transverse actin helps to position the sites of secondary cell wall synthesis, a process that initiates the formation of xylem vessels (Wightman and Turner 2008). Thus, we hypothesize that the cytoskeletal proteins differentially accumulating in seminal and nodal roots might be involved in root-type-specific xylem development. We also found that the protein CS1 (protein ID: m0w2v6) was detected exclusively in nodal roots (Fig. 2D, Supplementary Table S5). Interestingly, this protein orchestrates cell wall synthesis alongside microtubules during the initial developmental phase of xylem vessel formation (Derbyshire et al. 2015, Schneider et al. 2017). In Arabidopsis, cs1 mutants exhibit reduced cellulose content and decreased cell length (Gu et al. 2010), which lead to stem torsion and twists (Landrein et al. 2013). In addition to CS1, also SPIRAL1-like microtubule-stabilizing factor (protein ID: f2e2m2) was present only in nodal roots (Fig. 2D, Supplementary Table S5). This is a plant-specific protein localized to microtubules within the cortical array and has been associated with cell elongation (Nakajima et al. 2004). However, whether CS1 and SPIRAL1-like microtubule-stabilizing factor are also involved with xylem development has not yet been investigated.

Another level of regulation in xylem formation and development is determined by the interplay between auxin and cytokinins, which is central in defining the protoxylem vessel domain (Bishopp et al. 2011, De Rybel et al. 2014). Although little is known about the regulation of metaxylem vessel formation, inhibition of auxin biosynthesis leads to metaxylem defects (Ursache et al. 2014), while auxin application restores xylem development (Digby and Wareing 1966). Thus, auxin promotes the differentiation of metaxylem vessels. Interestingly, we detected higher concentrations of IAA in nodal roots than in seminal roots (Fig. 3B), raising the possibility that these higher levels are causally related with a larger number of metaxylem vessels formed in nodal roots (Fig. 1K). Despite their central role in protoxylem vessel specification (Kondo et al. 2011, Ren et al. 2013), the role of cytokinins in metaxylem formation remains unclear. While the active forms of cytokinins were below our detection limits, the levels of the corresponding riboside forms were not significantly different between seminal and nodal roots (Fig. 3A). Thus, we speculate that the higher levels of auxin might be involved with the enlarged metaxylem area and larger number of metaxylem vessels in nodal roots. However, it remains unclear whether the high auxin levels detected in nodal roots are due to enhanced local auxin biosynthesis or increased rootward flow of shootborne auxin.

**A role of nodal root anatomy in nitrate uptake and translocation**

Although anatomical differences between root types have been documented in different cereal species, the consequence of these differences for plant growth and physiology has remained elusive. The major function of xylem vessels is the long-distance transport of water and nutrients from the root to the shoot. Therefore, a larger diameter and number of metaxylem vessels could potentially enhance the transport capacity of water and nutrients (Burton et al. 2013). During domestication, maize landraces tended to retain longer nodal roots and larger xylem vessel areas as compared to teosintes (Burton et al. 2013). Thus, we speculate that the selection of these traits during domestication could be associated with the practices of irrigation and fertilization in farming systems.

In rootstock of the citrus species *Poncirus trifoliata*, a high correlation between root hydraulic conductivity and the diameter of xylem vessels has been found (Rodríguez-Gamir et al. 2010). Similar results were also observed in avocado plants, in which plants with 19% larger xylem area had 29% higher xylem sap flow rates (Fassio et al. 2009). In a genetic approach, it has been demonstrated that knocking out *XND1* leads to increased xylem number and area, which resulted in higher root hydraulic conductivity in Arabidopsis (Tang et al. 2018). Previously, it has been shown that nodal roots of barley exhibit a higher hydraulic conductivity than seminal roots (Knipfer et al. 2011), but the reason behind was not known. Our anatomical analysis suggests that these differences might be associated with the increased number of metaxylem vessels and the enlarged xylem area of nodal roots (Fig. 1J, K). Also, in maize, crown roots with larger metaxylem number were associated with higher hydraulic conductivity (Tai et al. 2016) and water uptake capacity (Ahmed et al. 2018) as compared to seminal roots. However, a relation between metaxylem anatomy and nutrient uptake or translocation has not yet been
reported. Unfortunately, in some of these studies, the developmental stage and tissue age of the different root types were not taken into account. Thus, additional studies are still necessary to directly investigate the relation between differences in xylem structure and hydraulic conductivity.

Under special consideration of the tissue age and developmental stage of different root types, we found no significant difference in nitrate uptake capacity between seminal and nodal roots of similar organ age, when nitrate was supplied at micromolar concentrations in the nutrient solution (Fig. 4A). However, at millimolar levels of external nitrate, nodal roots had almost 2-fold higher nitrate uptake and root-to-shoot translocation rates than seminal roots (Fig. 4B, C). These large differences were hardly related to the expression levels of low-affinity nitrate transporters, as only one nitrate transporter (NRT1.1 type; HORVU7H1R1G071600) was significantly higher expressed in nodal roots (Fig. 5B). Instead, we propose that the greater metaxylem area and increased number of metaxylem vessels facilitated more nitrate transport into and through nodal roots. Our attempt to verify larger root-to-shoot translocation capacities also for Rb<sup>+</sup> or Sr<sup>2+</sup> failed (Supplementary Fig. S5), probably because uptake and translocation rates were much lower than for nitrate, thus causing translocation rates no longer being favored by higher xylem conductivity in nodal roots. Notably, external N conditions can alter root anatomy. In fact, high N supply increased the stele radius of fine roots in trees (Wang et al. 2018), whereas low N reduced the total vessel area of maize roots in both seminal and nodal roots, as well as the number of metaxylem in nodal roots (Gao et al. 2015). Interestingly, in maize, high nitrate supply increases auxin levels in the stele of nodal roots (Yu et al. 2015b). This could, in turn, induce metaxylem formation since auxin positively regulates metaxylem development (Ursache et al. 2014). In the case of poplar, high N availability has been shown to enhance xylem diameter, which translated into altered hydraulic properties with increased xylem transport efficiency (Plavcova et al. 2013). These findings suggest a positive impact of external nitrate on xylem development and nitrate transport. In addition to anatomy, we found elevated sucrose levels in nodal compared to seminal roots (Fig. 4D). In Arabidopsis, increased sucrose supply leads to enlarged root diameter and enhanced nitrate uptake (Lejay et al. 1999, Lee-Ho et al. 2007). This is in agreement with our observations of a larger root diameter and elevated nitrate uptake in nodal roots (Figs. 4, 11, Supplementary Fig. S5). However, it also worth noting that the higher sucrose levels detected in nodal roots (Fig. 4D) could indicate that these roots represent a larger sink and require higher metabolic costs than seminal roots. This could explain why under low N availability, maize plants with reduced crown root numbers are metabolically more efficient for N acquisition (Saengwilai et al. 2014).

Taken together, our study suggests that nodal roots are not only more efficient than seminal roots for water uptake, as shown by previous studies, but also for nitrate uptake and long-distance transport, especially when external resources are adequate. These findings emphasize the potential and the need to further explore root anatomical traits that may help improving water and N uptake by selecting lines with beneficial anatomical root traits.

**Materials and Methods**

**Plant culture**

Barley seeds, cv. Golden Promise, were germinated on wet filter paper for 5 d at 4°C in the dark. Germinated seedlings were transferred to half-strength nutrient solution without Fe supply for 7 d in a climate chamber under short-day conditions at a 20/18°C and 10/4-h light–dark regime and a light intensity of 250 μmol m<sup>−2</sup> s<sup>−1</sup> at 70% humidity. Then, seedlings were transferred to full nutrient solution, containing 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM K<sub>2</sub>SO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 0.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1 mM KCl, 1 μM H<sub>3</sub>BO<sub>3</sub>, 0.5 μM MnSO<sub>4</sub>, 0.5 μM ZnSO<sub>4</sub>, 0.2 μM CuSO<sub>4</sub>, 0.01 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> and 0.1 mM Fe-EDTA. With plant transfer to hydroponics, conditions were switched to long day following a 20/18°C and 16/8-h light–dark regime with a light intensity of 250 μmol m<sup>−2</sup> s<sup>−1</sup> at 70% humidity. The nutrient solution was replaced every 3–4 d. Seminal and nodal roots of an approximate similar age defined as 50–60 cm long, were harvested at 25 or 39 d after sowing, respectively. Samples for the analysis of 15N, sugars, hormones, proteomic profiling and qRT-PCR were frozen in liquid N immediately after harvest.

**Analysis of morphological root traits and microscopy**

Fresh plant tissues were photographed under a VHX-5000 digital microscope (Keyence Neu-Isenburg, Germany). Segments of the root mature zone were embedded in 4% agar and sliced by a vibratome (VT1000S; Zeiss) and photographed under a light microscope (Axio Imager 2; Zeiss). Areas of different cell types were quantified by ImageJ.

**15N uptake and translocation**

To assess the high-affinity uptake capacity of both root types, plants were first exposed to N starvation solution for 2 d. All nutrients were maintained as mentioned above, except that 2 mM Ca(NO<sub>3</sub>)<sub>2</sub> was replaced by 2 mM CaCl<sub>2</sub>. Plant roots were rinsed in 1 mM CaSO<sub>4</sub> solution for 1 min, and then 50 μM 15N-labeled KNO<sub>3</sub> (98 atom% 15N, Sigma) for 5 min. Roots were washed with 1 mM CaSO<sub>4</sub> to remove apoplastic 15N, separated from shoots and stored at −80°C before freeze drying.

In the case of low-affinity uptake capacity of both root types, plants were first exposed to N starvation solution for 2 d. All nutrients were maintained as mentioned above, except that 2 mM Ca(NO<sub>3</sub>)<sub>2</sub> was replaced by 2 mM CaCl<sub>2</sub>. Plant roots were rinsed in 1 mM CaSO<sub>4</sub> solution for 1 min, and then 50–60-cm-long seminal or nodal roots were transferred to full nutrient solution containing 200 μM 15N-labeled KNO<sub>3</sub> (98 atom% 15N, Sigma) for 5 min. Roots were washed with 1 mM CaSO<sub>4</sub> to remove apoplastic 15N, separated from shoots and stored at −80°C before freeze drying.

**Sugar, hormone and protein quantification**

For sugar analysis, samples were incubated for 60 min at 80°C in 80% ethanol and centrifuged for 15 min at 14,000 rpm. Supernatants were evaporated to dryness at 45°C and then dissolved by MQ water. Dissolved samples were subjected to the enzyme-coupled photometric assay, as described in Höller et al. (2014).

Hormone quantification was carried out following the procedure described by Eggert and von Wirén (2017). Briefly, about 20 mg freeze-dried root samples were subjected to 0.1% formic acid extraction. Samples were centrifuged for 10 min at 4°C and 17,500 × g. The remaining pellets were re-extracted, and both supernatants were combined. The 50-μl internal standard mix including auxins and cytokinins was added to each supernatant. Supernatant was then added to
an 1 cc/30 mg HLB cartridge (Waters, Milford, MA, USA). Auxins were separated from the basic cytokinins by using a 1 cc/30 mg MCX cartridge (Waters). Eluted solutions were subjected to LC–MS–MS (Waters) quantification.

To compare their proteome, five biological replicates of each root type were subjected to protein extraction according to the phenol extraction/ammonium acetate precipitation adapted for gel-free proteomics. After protein quantification (2-D Quant Kit; GE Healthcare), 20 μg of proteins were digested with trypsin (Trypsin Protease, MS Grade; Thermo Scientific, USA) followed by purification with Pierce C18 Spin Columns (Thermo Scientific). The digested samples (0.5 μg/5 μl) were separated in an Ultimate 3000 UPLC system (Thermo Scientific) and then in a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) as described previously (Campos et al. 2016). The Q Exactive Orbitrap mass spectrometer (Thermo Scientific) was operated in positive ion mode with a nano spray voltage of 1.5 kV and a source temperature of 250°C. ProteoMass LTQ/FT-Hybrid ESI Pos. Mode Cal Mix (MS CALS1EASUPELCO; Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. The instrument was operated in a data-dependent acquisition mode with a survey MS scan at a resolution of 70,000 (full width at half maximum at m/z 200) for the mass range of m/z 400–1,600 for precursor ions, followed by MS/MS scans of the top 10 most intense peaks with +2, +3, +4 and +5 charged ions above a threshold ion count of 16,000 at 17,500 resolution using the normalized collision energy of 25 eV with an isolation window of 3.0 m/z and a dynamic exclusion of 10 s. All data were acquired with Xcalibur 3.0.63 software (Thermo Scientific). For protein identification, we used Mascot version 2.2.06 (Matrix Science) against uniprot database Hordeum vulgare (74,028 entries, containing the usual mass spec contaminants) (https://www.uniprot.org/). The false discovery rate (FDR) was calculating using Scaffold 3 (Proteome Software Inc.). The parameters used to search were: parent mass tolerance of 10 PPM, fragment tolerance of 0.02 Da, oxidation of M as variable modification, carbamidomethyl C as fixed modification and up to one missed cleavage was allowed for trypsin. For identification and alignment, all raw data were converted into mgf- files using Progenesis (v 4.1; Nonlinear Dynamics, UK). For GO term analysis, selected proteins were subjected to agriGO online tool (http://bioinfo.cau.edu.cn/agriGO/) for enrichment approach with default parameters except ‘Plant GO slim’ was chosen as the GO type (Tian et al. 2017).

**Real-time quantitative PCR**

Total RNA was extracted using the QiAzoLyT M Lysis reagent (Qiagen) following the manufacturer’s instructions. Prior to cDNA synthesis, samples were treated with DNase (Thermo Fisher Scientific). Reverse transcription was performed using SuperScript™ II (Thermo Fisher Scientific) reverse transcriptase and Oligo. Real-time PCR by was conducted using a Mastercycler ep realplex (Eppendorf) and QuantiTect SYBR Green qPCR mix (QIAGEN). Gene-specific primer pairs are listed in [Supplementary Table S6](#). Primer specificity was confirmed by the analysis of melting curves. Relative expression levels were calculated according to Pfaffl (2001). Barley UBQUITIN C (AY220735.1) was taken as reference gene.

**Statistical analysis**

All statistical analyses were performed using SigmaPlot 11.0. Mean values were compared by Student’s t-test (P < 0.05).

**Supplementary Data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


